Available online at www.sciencedirect.com
SCIENCE DIRECT®
Food Chemistry 93 (2005) 599–605
Food Chemistry
www.elsevier.com/locate/foodchem

Contents and antioxidant capacity of limonin and nomilin in different tissues of citrus fruit of four cultivars during fruit growth and maturation

ChongDe Sun [a], KunSong Chen [a,*], Yang Chen [a], QingJun Chen [b]

[a] *Laboratory of Fruit Molecular Physiology and Biotechnology/The State Agriculture Ministry Laboratory of Horticultural Plant Growth, Development and Biotechnology, Zhejiang University, Huajiachi Campus, Hangzhou 310029, PR China*
[b] *Food Inspection Station of Zhejiang Province, Hangzhou 310009, PR China*

Received 7 June 2004; received in revised form 5 October 2004; accepted 5 October 2004

Abstract

The contents of limonin and nomilin in different fruit tissues of Foxiangyou (*Citrus grandis*), *Citrus unshiu*, Penggan (*Citrus reticulata*) and Huyou (*Citrus changshanensis* KS Chen et CX Fu) were measured during fruit growth and maturation by HPLC (high performance liquid chromatography). Results showed that limonin and nomilin were the predominant limonoids in the extracted samples. During fruit growth and maturation, the contents of limonin and nomilin increased from April, peaked in early September and decreased afterwards until late October when they reached a steady low level. The antioxidant capacities of limonin and nomilin in the four tissues of mature fruit were determined by β-carotene bleaching assay. The results showed that the antioxidant capacities of limonin and nomilin varied in different tissues and cultivars. In the three tissues other than albedo, the antioxidant capacities of limonin and nomilin were high (2.9–8.3 times than that of vitamine C).

Keywords: Limonin; Nomilin; Citrus; Antioxidant capacity

1. Introduction

Many diseases, such as cancer, atherosclerosis and inflammation are caused by free radicals and lipid peroxidation inside human bodies. This kind of risk can be reduced by an appropriate dietary pattern including a great portion of fruit and vegetables (Ames, Shigenaga, & Hagen, 1993; Weisburger, 1999) because of the great amount of natural antioxidants in these plant foods (Cheung, Cheung, & Ooi, 2003).

Limonoids, first reported in 1864 (Emerson, 1948), are a group of chemically related triterpene derivatives found in the Rutaceae and Meliaceae families (Bennett & Hasegawa, 1982; Hashinaga, Fong, & Hasegawa, 1990). Many studies have shown that limonoids present in citrus are biologically active, displaying anticarcinogenic activity, abirritation and antifeedant activity against insects (Bentley, Rajab, Alford, Mendel, & Hassanali, 1988; Lam, Li, & Hasegawa, 1989). It is reported that dietary supplementation with the citrus limonoids, limonin and nomilin, activates glutathione *S*-transferase in the liver and small intestine of the rat (Kelly, Jewell, & O'Brien, 2003), suppresses carcinogenesis (Lam et al., 1989; Miller et al., 1989; Miyake, Ozaki, & Bennett, 1992). In vitro limonin, nomilin and limonoid glucosides (LG) were proved to have a significant ability to inhibit proliferation of human breast cancer. Nomilin was the most effective followed by limonin and LG (Karim & Hashinaga, 2002; Kelly et al., 2003).

* Corresponding author.
E-mail address: akun@zju.edu.cn (KunSong Chen).

0308-8146/$ - see front matter © 2004 Elsevier Ltd. All rights reserved.
doi:10.1016/j.foodchem.2004.10.037

Citrus plants from the Rutaceae family are the main source of the natural liminoids and 39 liminoid aglycones and 21 glucosides have been isolated from citrus plants so far (Ohta, Berhow, Bennett, & Hasegawa, 1992). Limonin and nomilin are the most prevalent citrus limonoids (Kelly et al., 2003).

So far, the studies of limonoids have been mainly on their chemical structures, varieties and sources with few about their content changes in different tissues and cultivars during maturation. By using *Citrus grandis*, *Citrus unshiu*, *Citrus reticulata* and *Citrus changshanensis* as materials, we studied the changes of limoinoid contents in different tissues and cultivars during fruit growth and maturation as well as the antioxidant capacities of limonin and nomilin in mature fruit. Hopefully, the results will prove useful for the utilization of limonoids in citrus fruit.

2. Materials and methods

2.1. Materials and chemicals

Experimental materials were the flavedo, albedo, segment membrane (SM) and juice vesicle of *C. changshanensis*, *C. grandis*, *C. unshiu*, and *C. reticulata*. The citrus fruit were obtained from Quzhou Citrus Institute in Zhejiang, China from April 21 to November 18, 2002 with samplings every 3 weeks. The citrus fruit was divided into tissues of flavedo, albedo, SM and juice vesicle, which were then separately dried at 45 °C and ground.

Limonin, nomilin, linoleic acid, β-carotene and Vc as standard samples were from Sigma Chemical Co.

2.2. Extraction of limonin and nomilin

3 g of powders from fruit samples were placed in a Soxhlet extractor, treated with 60 ml CH_2Cl_2 and then extracted at 50 °C for about 60 min. The extract was filtered and the filtered solution was evaporated, close to dryness at about 30 °C. The residue was then dissolved in CH_2Cl_2 and moved to another vaporizer and the solution was then evaporated to dryness. The residue was dissolved in a minimal portion of acetonitrile (1 ml) and transferred to an Eppendorf tube (1.5 ml). The solution was filtered using a micro-filter (∅13, organism, aperture 0.30 μm). A part of the filtered solution was for the HPLC analysis and an other was used to determine the antioxidant capacity.

2.3. Determination of limonin and nomilin by TLC and HPLC

The identification of limonin and nomilin was done by retention time with HPLC, assisted by R_f with TLC, and the determination was made by the peak area (HPLC).

The silica gel for thin-layer chromatography was 60H and the plate was 150 mm × 100 mm. The solutions of standard and sample of limonin and nomilin were applied onto the TLC plate at the same height and then developed by the mobile phase, consisting of dichloromethane: acetic ether (4:7) in a tank which contained the mobile phase; a run distance of 13.5 cm was utilized. After developing, the TLC plates were dried and sprayed with *p*-dimethylaminobenzalrhodamine:sulfuric acid:ethanol(1:2:97(w/w)) in a ventilated closet. Then the plate was dried and heated at 125 °C for 5–8 min; then the colour appearing allowed R_f values to be detected.

20 μl sample solutions of limonin and nomilin were injected into the America Beckman HPLC (equipped with 125 pump, 166 UV monitor, Beckman System Gold control system and data processor). The HPLC analysis included a C_{18} column (4.3 mm × 25 cm), a mobile phase of methanol: acetonitrile: PBS (containing 0.03 mol/l of postassium dihydrogan phospate, pH 3.5) = 10:40:39 (v/v), flow rate of 1.0 ml/min and UV wavelength at 210 nm, and elution was carried out at room temperature (25 °C).

2.4. Determination of antioxidant capacity

The antioxidant activity of fruit extracts was determined by the method of β-carotene bleaching. A reagent mixture, containing 0.5 ml of β-carotene (Sigma) solution (0.2 mg/ml in chloroform), 0.01 ml of linoleic acid (Sigma), and 0.1 ml of Tween 80 (Sigma) was evaporated to dryness under a nitrogen stream in a 300 ml tube; 25 ml of oxygenated distilled water and 0. 5 ml of crude fruit extracts of limonin and nomilin from different tissues were added. Pure water, or acetonitrile (0.2 ml), was used as the control, Vc solution(2 mg/ml in water and acetonitrile = 1:4) used as standard, and the blank contained all the earlier chemicals except β-carotene. All these mixtures were then shaken to form a liposome solution and then incubated at 50 °C in water for 2 h. The absorbance of an aliquot (3 ml) of these liposome solutions at 470 nm was monitored by a spectrophotometer (UV 2001, Japan) at time a interval of 20 min. All samples were assayed in triplicate. The relative antioxidant activity was calculated according to the following equation:

$$\mathrm{AAC} = \frac{A_{S(120)} - A_{C(120)}}{A_{C(0)} - A_{C(120)}} \times 1000,$$

where $A_{S(120)}$ is the absorbance of sample at time 120, $A_{C(120)}$ is the absorbance of standard at time 120, $A_{C(0)}$ is the absorbance of standard at time 0.

2.5. Statistical analysis

The data were analyzed by DPS (Data Processing System) and Microsoft Origin software.

3. Results

3.1. Determination by TLC and HPLC

In many reports, the qualitative and quantitative detection of limonin and nomilin were done only by HPLC (Herman, Fong, Ou, & Hasegawa, 1990; Rouseff & Fisher, 1980) or only by TLC (Maier & Grant, 1970). In our research, we used HPLC assisted by TLC to detect limonlin and nomilin and HPLC to determine their contents quantitatively. The data showed that the results of these two methods were consistent (Figs. 1 and 2).

Limonin and nomilin were the main components with few impurities (Fig. 2). The recycling rates of limonin and nomilin were 90.4% and 90.1%, respectively. They show a good linear relationship (0.05–0.25 mg/g). The linear regression equations were: $A_L = 1.2478 + 0.21208C_L$ ($r = 0.996$), $A_N = 0.0864 + 0.128563C_N$($r = 0.999$); Detection limit ($S/N = 3$) = 0.03 μg/g; RSD ($n = 5$) = 6.48%, 2.90%, respectively.

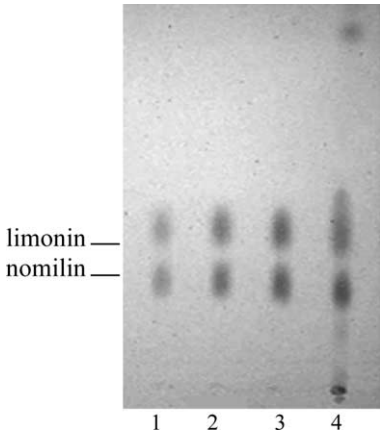

Fig. 1. TLC of limonin, nomilin for the sample extracted from segment membrane of Zhoushan pumelo 1–3: standard sample amount of limonin and nomilin, respectively (1.25, 1.75, 2.25 μg), 4 sample extracted from segment membrane (5.0 μl).

3.2. Contents of limonin and nomilin in different fruit tissues of four citrus cultivars during fruit growth and maturation

3.2.1. General contents

The contents of limonoate A-ring lactone (LARL) and LG in pericarp and juice vesicle tissues of navel orange fruit have been reported (Hasegawa et al., 1991). In the current work, we determined the limonin and nomilin contents in the tissues of flavedo, albedo, SM and juice vesicles of different citrus cultivars, including *C.grandis*, *C. unshiu*, *C. reticulata and C. changshanens*. Generally, the contents of limonin and nomilin first increased, and gradually decreased afterwards, during the whole period of growth and maturation. But in albedo of *C. grandis*, both limonin and nomilin contents changed little and remained at a quite low level.

3.2.2. Flavedo

In flavedo, the nomilin content in *C. grandis* and the limonin content in *C. reticulata* changed most markedly. But after maturation, the nomilin content in *C. grandis* decreased to a non-detectable level while the limonin contents in *C. reticulata* and *C. changshanensis* were still quite high, which were 0.76 and 0.796 mg/g (DW), respectively. In *C. unshiu*, both nomilin and limonin contents changed little and remained at low levels (Fig. 3).

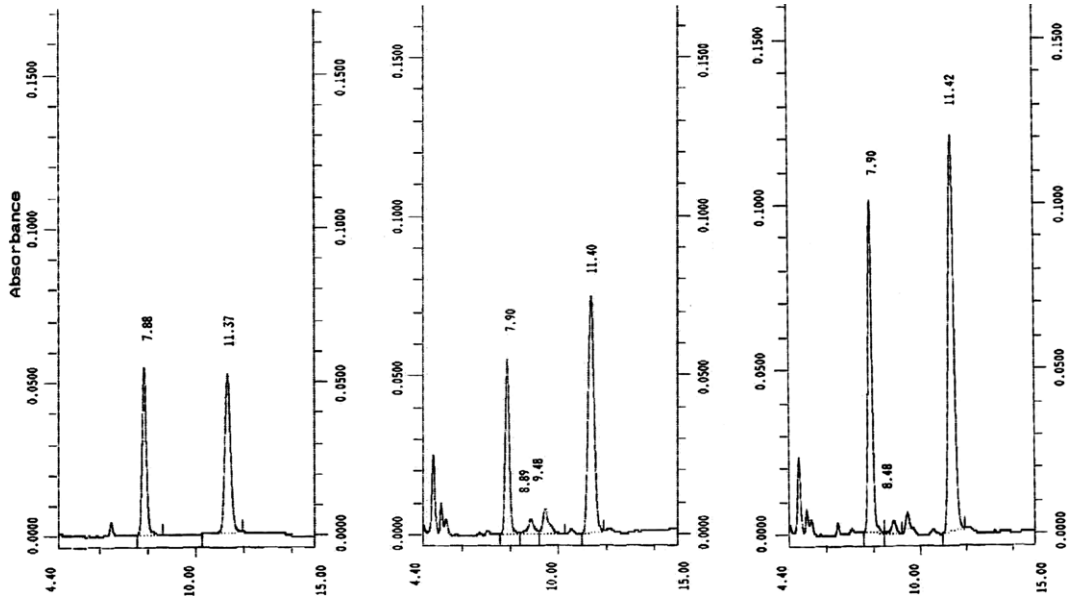

Fig. 2. HPLC of limonin, nomilin and the sample extracted from citrus fruit (A standard B sample C sample and standard).

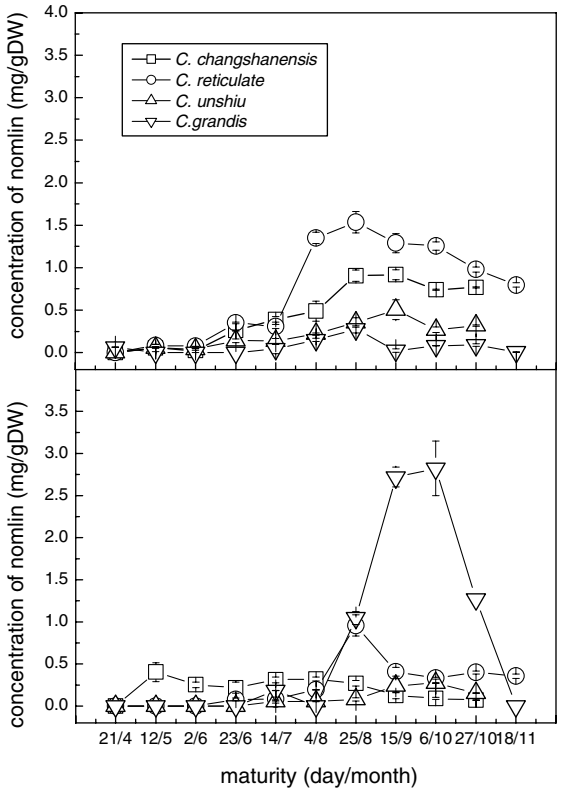

Fig. 3. Changes in the limonin and nomilin contents of flavedo during fruit development.

3.2.3. Albedo

In albedo of *C. changshanensis*, both limonin and nomilin concents were constantly high, throughout the growth, and they were 0.578 and 0.635 mg/g (DW) when the fruit reached maturation. In *C. unshiu* and *C. reticulata*, their contents changed in a pattern similar to *C. changshanens.* But in albedo of *C. grandis*, both contents changed little and remained low (Fig. 4).

3.2.4. Segment membrane

Limonin and nomilin contents in Segment membrane (SM) of *C. changshanensis* remained high with maxima of 3.52 and 2.94 mg/g (DW), respectively, and, even after maturation, their contents still reached 0.524 and 0.767 mg/g. In *C. unshiu* and *C. reticulata*, their contents changed similarly. At the end of fruit development, there was an increase of the limonin and nomilin contents in SM of *C. grandis* and especially the nomilin content increased quite obviously (Fig. 5).

3.2.5. Juice vesicle

In juice vesicle of all the cultivars, the limonin and nomilin contents were all quite low. They remained at a quite low levels throughout the growth and the trend of changes was similar to that in SM. In juice vesicle of mature fruit, the limonin content in *C. changshanensis* was the highest and in *C. grandis*, the nomilin content was the highest (Fig. 6).

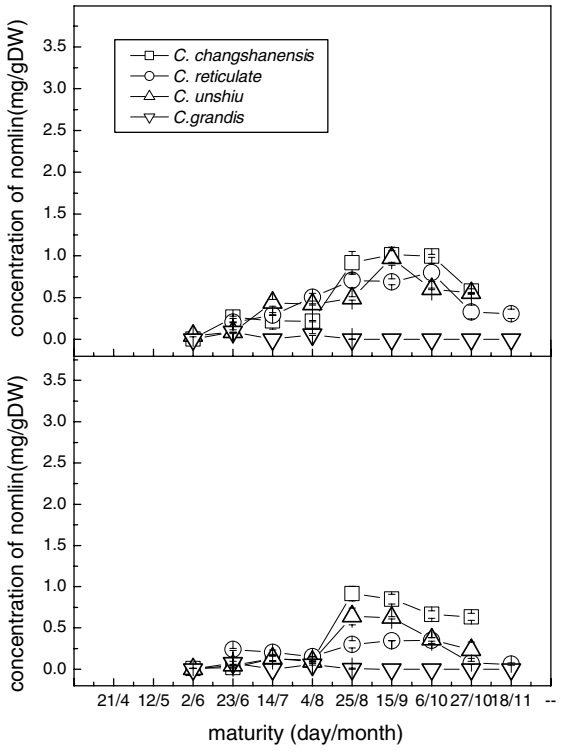

Fig. 4. Changes in the limonin and nomilin contents of albedo during fruit development.

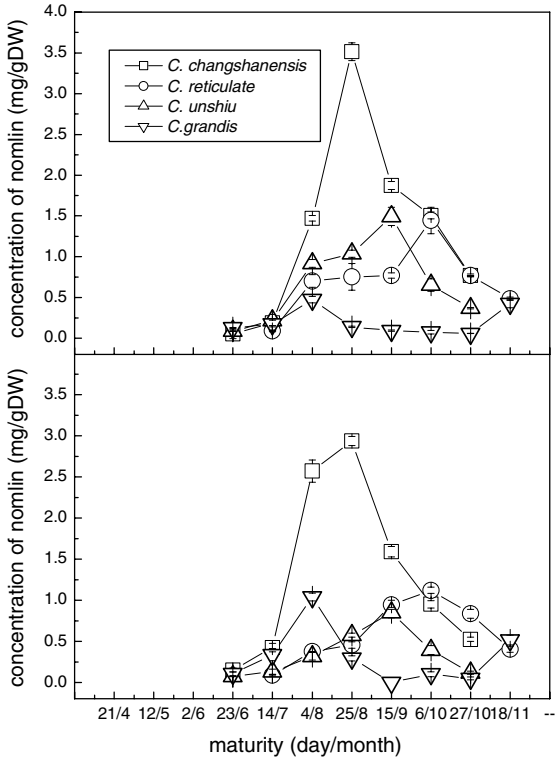

Fig. 5. Changes in the limonin and nomilin contents of segment membrane during fruit development.

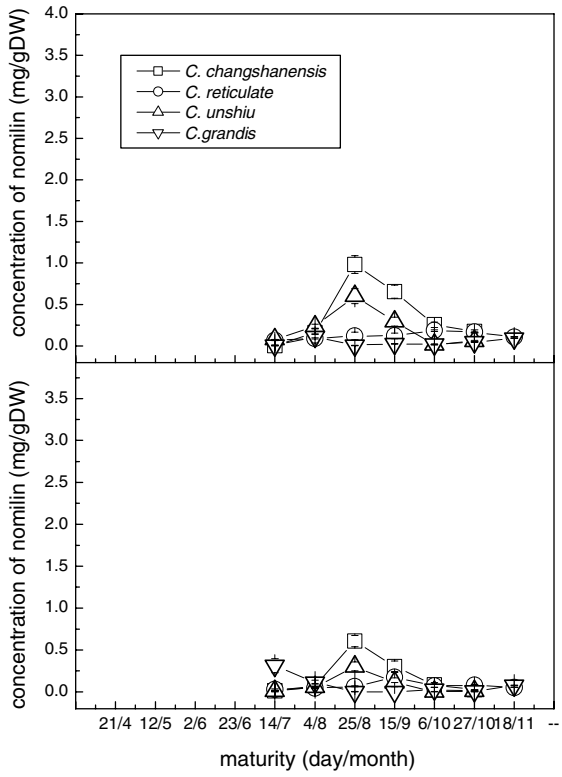

Fig. 6. Changes in the limonin and nomilin contents of juice vesicle during fruit development.

Generally speaking, in albedo and SM, it was *C. changshanensis* and in flavedo, it was *C. reticulata* that had the highest total content of limonin and nomilin. In juice vesicle, the total contents of limonin and nomilin in *C. changshanensis*, *C. reticulata* and *C. grandis* did not show a significant difference (Table 1).

Table 1
Total contents of limonin and nomilin in different tissues of mature fruit of the four cultivars (mg/g (DW))

	Flavedo	Albedo	SM	Juice vesicle
C. changshanensis	0.835de	1.21b	1.29ab	0.197h
C. reticulata	1.35a	0.369g	0.890d	0.164h
C. grandis	0.0067i	0.0037i	1.01c	0.168h
C. unshiu	0.472f	0.784e	0.484f	0.068i

Different letters refer to significant differences ($\alpha = 0.05$).

The results showed that, at the end of fruit development, the contents of limonin and nomilin noticeably decreased, which is probably because of the conversion of limonoids to the corresponding glucosides (Hasegawa, Bennett, Herman, Fong, & Ou, 1989; Hasegawa et al., 1991; Ozaki et al., 1991).

3.3. Antioxidant capacities of limonin and nomilin

Many artificial antioxidants (BHA, BHT, TBHQ) have been commonly used as food additives (Cheung et al., 2003). However, the artificially synthesized BHA and BHT may lead to cancer (Botterweck, Verhagen, Goldbohm, Kleinjans, & Brandt, 2000). Consequently, the development of natural antioxidants has become important in the field of food science. In our work, the antioxidant capacities of limonin and nomilin in the four tissues of mature fruit of the four cultivars were determined by β-carotene bleaching assay.

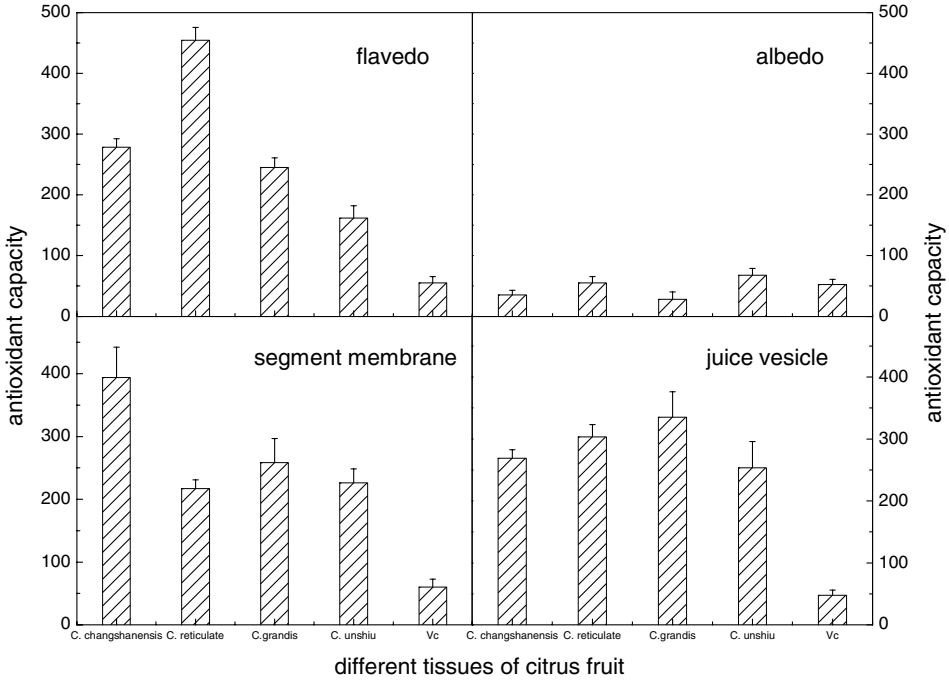

Fig. 7. Antioxidant capacity of limonin and nomilin in different tissues of fruit.

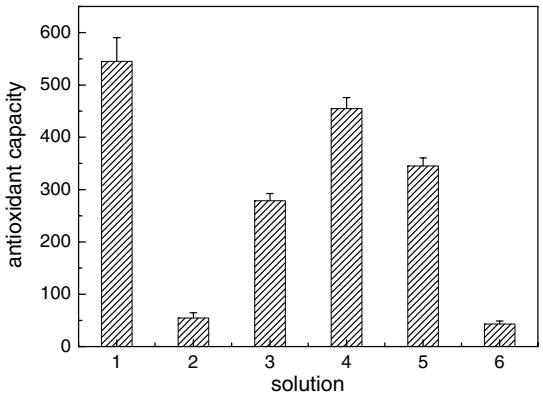

Fig. 8. Antioxidant capacity of limonin and nomilin standard and the sample from citrus fruit tissues: 1. 50 μg limonin standard, 2. 50 μg nomilin standard, 3. 25 μg standard limonin + 25 μg standard nomilin, 4. 50 μg sample, 5. 12.5 μg standard imonin + 12.5 μg standard nomilin + 25 μg sample, 6. 50 μg Vc.

In citrus fruit, the antioxidant capacity of limonin and nomilin varied much in different tissues and cultivars. In the three tissues other than albedo, the antioxidant capacities of limonin and nomilin were quite high, which was markedly higher than that of Vc ($P < 0.01$). In flavedo, the antioxidant capacities of limonin and nomilin were highest in *C. reticulata*. In SM, they were highest in *C. changshanensis* and in juice vesicle, highest in *C. grandis.* Although the limonin and nomilin contents were low in juice vesicle (0.0680–0.19 mg/g), the antioxidant capacities were quite high, showing that eating juice vesicle is good for health. The antioxidant capacities of limonin and nomilin in albedo of all the cultivars were all quite low and do not show a significant difference from that of Vc.

In order to certify the antioxidant capacities of limonin and nomilin in the sample from citrus fruit tissues, we compared our sample with the standard limonin and nomilin. The result showed that the antioxidant capacity of a 50 μg sample was a little lower than that of a 50 μg limonin standard, but it was markedly higher than that of 50 μg nomilin standard or 25 μg limonin + 25 μg nomilin standard. As to the standard limonin and nomilin, the antioxidant capacity of limonin was 12.7 times higher than that of nomilin (Figs. 7 and 8). It is obvious that both the standard limonin and nomilin and the limonin and nomilin in the sample from citrus fruit tissues have antioxidant capacity, and limonin and nomilin were the leading antioxidants in the sample.

Acknowledgements

We acknowledge Mr Chunrong Liu, Director of Quzhou Citrus Research Institute, Zhejiang; Mr Peilin Fang, Deputy Director of Agricultural Bureau of Kecheng District, Quzhou, Zhejiang; and Mr Xingjiang Qi, Deputy Director of Horticultural Institute of Zhejiang Academy of Agricultural Sciences for their great help and support. The work was supported by the state Key Basic Research and Development Plan (6-Z000046806), the National Natural Science Foundation of China (30170660) and Zhejiang Natural Science Foundation (ZD0004).

References

Ames, B. N., Shigenaga, M. K., & Hagen, T. M. (1993). Oxidants, antioxidants, and the degenerative diseases of aging. *Proceedings of the National Academy of Sciences of the United States of America, 90*, 7915–7922.

Bennett, R. D., & Hasegawa, Shin. (1982). 7α-Oxygenated limonoids from the rutaceae. *Phytochemistry, 21*, 2349–2354.

Bentley, M. D., Rajab, M. S., Alford, A. R., Mendel, M. J., & Hassanali, A. (1988). Structure-activity studies of modified citrus limonoids as antifeedants for Colorado potato beetle larvae, Leptinotarsa decemlineata. *Entomologia Experimenta is et l Applicata, 49*, 189–193.

Botterweck, A. A. M., Verhagen, H., Goldbohm, R. A., Kleinjans, J., & Brandt, P. A. (2000). Intake of butylated hydroxyanisole and butylated hydroxytoluene and stomach cancer risk: results from analyses in the Netherlands cohort study. *Food and Chemical Toxicology, 38*, 599–605.

Cheung, L. M., Cheung, P. C. K., & Ooi, V. E. C. (2003). Antioxidant activity and total phenolics of edible mushroom extracts. *Food Chemistry, 81*, 249–255.

Emerson, O. H. (1948). The bitter principles of citrus fruit. *Journal of the American Chemical Society, 70*, 545–549.

Karim, M. R., & Hashinaga, F. (2002). Isolation and characterization of limonoid glucosyltransferase from pummelo albedo tissue. *Food Chemistry, 76*, 431–436.

Hasegawa, S., Bennett, R. D., Herman, Z., Fong, C. H., & Ou, P. (1989). Limonoid glucosides in citrus. *Phytochemistry, 28*, 1717–1720.

Hasegawa, S., Ou, P., Fong, C. H., Herman, Z., Coggins, C. W., Jr., & Atokin, D. R. (1991). Changes in the limonate A-ring lactone and limonoid 17-β-glucopyranoside content of navel oranges during fruit growth and maturation. *Journal of Agricultural and Food Chemistry, 39*, 262–265.

Hashinaga, F., Fong, C. H., & Hasegawa, S. (1990). Biosynthesis of limonoids in citrus sudachi. *Agricultural Biology Chemistry, 54*, 3019–3020.

Herman, Z., Fong, C. H., Ou, P., & Hasegawa, S. (1990). Limonoid glucosides in orange juices by HPLC. *Journal of Agricultural and Food Chemistry, 38*, 1860–1861.

Ohta, H., Berhow, M., Bennett, R. D., & Hasegawa, S. (1992). Limonoids in seeds of citrus hanaju. *Phytochemistry, 31*, 3905–3907.

Kelly, C., Jewell, C., & O'Brien, N. M. (2003). The effect of dietary supplementation with the citrus limonoids, limonin and nomilin on xenobiotic-metabolizing enzymes in the liver and small intestine of the rat. *Nutrition Research, 23*, 681–690.

Lam, L. K. T., Li, Y., & Hasegawa, S. (1989). Effects of citrus limonoids on glutathione S-transferase activity in mice. *Journal of Agricultural and Food Chemistry, 37*, 878–880.

Maier, V. P., & Grant, E. R. (1970). Specinc thin-layer chromatography assay of limonin, a citrus bitter principle. *Journal of Agricultural and Food Chemistry, 18*, 250–252.

Miller, E. G., Fanous, R., Rivera-Hidalgo, F., Binnie, W. H., Hasegawa, S., & Lam, L. K. (1989). The effect of citrus limonoids on hamster buccal pouch carcinogenesis. *Carcinogenesis, 10*, 1535–1537.

Rouseff, R. L., & Fisher, J. F. (1980). Determination of limonin and related limonoids in citrus juice by high performance liquid chromatograghy. *Analytical Chemistry, 52*, 1228–1233.

Miyake, M., Ozaki, Y., & Bennett, R. D. (1992). Limonoid glucosides in calamomdin seeds. *Phytochemistry, 31*, 1044–1046.

Weisburger, J. H. (1999). Mechanisms of action of antioxidants as exemplified in vegetables, tomatoes and tea. *Food and Chemical Toxicology, 37*, 943–948.

Ozaki, Y., Fong, C. H., Herman, Z., Maeda, H., Miyake, M., Ifuku, Y., & Hasegawa, S. (1991). Limonoid glucosides in citrus seeds. *Agricultural Biology Chemistry, 55*, 137–141.